PATHOGENICITY OF *Beauveria bassiana* TO ADULT *Diaprepes abbreviatus*

By

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I would like to dedicate this work to the memory of my late mentor Dr. Everett Royal Mitchell. His support through my undergrad work, time in the Peace Corps and master's work will always be remembered. His work in insect biological control was a great inspiration.

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I would like to extend my thanks to Dr. J.W. Kimbrough for priceless guidance in mycology, good advice about private matters, and a good joke every now and again. I would also like to thank his wife, Jane, for many good home-cooked meals. I would also like to thank the members of my committee Dr. F.W. Zettler, Dr. C.W. McCoy, Dr. J.M. Sivinski, and Dr. K.L. Buhr, for their enthusiasm for this project as well as their input into its production into a final product. I would like to thank Dr. C.W. McCoy for his initial interest and input in this project; without his innovative ideas this project would not have been a success. Dr. Zettler's assistance in acquiring an academic fellowship will always be appreciated as well as his sense of humor and numerous letters of recommendation for various awards. Dr. K.L. Buhr was the reason I joined the Peace Corps after receiving my B.S.; his agreeing to serve as my external committee member was greatly appreciated.

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I am indebted to the Florida Department of Agriculture in Gainesville for a healthy supply of weevils. Suzanne Fraser was always available at a moment's notice to send insects and helpful hints. The constant and reliable supply made my research possible.

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The *Diaprepes* root weevil (DRW) is a serious insect pest in the state of Florida. It causes an estimated $75 million in damages every year to Florida's citrus industry. Attempts at chemical control of this pest are costly with limited effectiveness. The use of *Beauveria bassiana,* a common native entomopathogen as a biological control agent of *Diaprepes abbreviatus* could reduce pesticide usage in controlling this pest. A commercially produced dry powder formulation of *Beauveria bassiana* was used in this research to determine its affect on weevil adults. Conidia of *Beauveria bassiana* were found to germinate on the weevil exoskeleton at various rates depending on the body

region. Conidia located in inter-segmental areas of the abdomen and head germinated at twelve hours post-inoculation; on other areas of the abdomen, germination was first observed eighteen hours post-inoculation. Germination on the elytra occurred 30 hours post-inoculation and even then, germination was low. Internal ramification of hyphal bodies began to appear 72 hours post-inoculation. The time between cuticle penetration and the development of hyphal bodies in the hemolymph appeared to be delayed by an immune response of the hemocytes. Internal proliferation of hyphae rapidly consumed the insect body cavity causing death after as few as 6 days. Sporulation on the cadaver usually occurred two to three days after death under moist conditions. *Diaprepes* root weevils interact by touching in the wild. This touching is not limited to sexual contact, inter-sexual touching also occurs and is possibly a form of communication. Weevils transmitted conidia through both bisexual and same sex contact. Adult mortality from this contact was not significantly different between sexes. These data suggest that adult weevils can spread fungal conidia within a native population.

CHAPTER 1
INTRODUCTION

The use of the hyphomycete, *Beauveria bassiana* (Balsamo) Vuillemin (Fungi Imperfecti: Hyphomycetes: Moniliaceae), as a mycoinsecticide in the biological control of insects has a long and interesting history beginning with a role as a pathogen of an economically important insect. According to Ainsworth (1956), Agostino Bassi was one of the first people to experiment with insects infected with *Beauveria*. Bassi (1835) bred silkworms (*Bombyx mori* L.) in an attempt to simulate white muscardine disease, a cause of economic losses in the silk industry. White muscardine is a disease characterized by insect death followed by a whitening of the exoskeleton. He devised many schemes to induce the disease, including several attempts that failed at inducing disease in healthy insects.

Bassi (1835) was the first to publish his belief that muscardine disease was caused by "a plant of the cryptogamic kind, a parasitic fungus." In the late 18th century muscardine disease was referred to in France as "Muscardine du ver à soie" (Beauverie, 1911) and was given the classification *Beauveria* (Vuillemin, 1912). *Beauveria bassiana* was described by Beauverie first in 1911 then again in 1914 as *Botrytis bassiana*. Beauverie (1911, 1914) and Vullemin (1912) originally described *Beauveria bassiana* as follows: Hyphae slender, 1.5-2 μ in diameter, hyaline, septate: colonies flat, mealy, or finely pulverulent with a chalky appearance somewhat like the surface of a newly broken piece of chalk, white to pale cream on the surface, does not color the undersurface of

potato dextrose agar; phialides, variable in shape, ventricose to filamentous, develop on the main hyphal branches or on short branchlets at right angles to the main axis, branching may be repeated, forming globose heads; spores borne on zigzag sterigmata that extend from the tip of the phialides, globose, 2.4 μ in diameter (MacLeod, 1954). *Beauveria* is a classic example of sympodial conidiogenesis in the Hyphomycetes Teleomorphs are unknown in *Beauveria* but are likely to belong to the *Clavicipitaceae* (*Cordyceps*, *Torrubiella* and related genera) (Arx, 1986; Kirk et al., 2001). *Beauveria bassiana* was one of the first entomopathogens to be studied and was also one of the oldest known fossilized entomopathogens found in Dominican amber (Poinar and Thomas, 1984).

This description of *Beauveria bassiana* provided characteristics useful in the identification of the organism, helped to clarify many taxonomic errors, and pulled many synonyms into the genus *Beauveria* (MacLeod, 1954). The genus *Tolypocladium* had at one time been a synonym of *Beauveria* based on similar conidiogenous structures and similar, white, lanose colonies (von Arx, 1986). The biochemical data supporting this synonymy of this genus with *Beauveria* have been questioned (Mugnai et al., 1989). Additional biochemical and genetic work used rRNA polymorphism, isoenzyme variability, and morphoontogenic characters confirming that these genera were distinct (Rakotonirainy et al., 1991). For these reasons, *Tolypocladium* will not be considered a synonym of *Beauveria* in this work.

Some synonyms of *Beauveria bassiana* include *Botrytis necans, Botrytis stephanoderis* and *Beauveria laxa.* According to Petch, (1926) confusion about synonyms was often based on quantitative rather than qualitative data. Based on morphological and

biochemical characteristics, there are at least six species of *Beauveria*: *B. alba, B. amorpha*, *B. bassiana, B. brongniarti, B. velata* and *B. vermiconia* (Mugnai et al., 1989). The inclusion of a new species previously placed in the genus *Isaria* is under debate (von Arx, 1988).

Beauveria bassiana has been found around the world and in many habitats (Ferron, 1981; St Leger et al., 1992). This fungus helps regulate populations of different insects including coleopterous insects of the genera *Diabrotica, Colaspis* and *Maecolaspis* in soybean grown in Argentina and Brazil. For example, in Brazil the fungus achieves high prevalence in populations of *Aracanthus*, an important pest of beans (Sosa-Gomez et al., 1994), and in France, England, and Morocco it can be found on Sitonia weevils, a major pest of cultivated Fabaceae (Poprawski et al., 1988).

The conidia of *Beauveria* can be mass-produced in quantities sufficient for use in microbial control; conidia can be produced on a semi-solid bran medium similar to that used in the commercial production of *Bacillus thuringiensis* (Feng et al., 1994; Soares et al., 1979). Isolates from the United States, Canada and China have been collected and used in immunochemical characterization to identify a quality control program to ensure virulence of a strain (Tan and Ekramoddoullah, 1990). Although most *B. bassiana* isolates are morphologically indistinguishable, they vary in virulence in their respective hosts. Biochemical characterization and large-scale production of these virulent isolates are important for successful biological control (Zhang and Tan, 1987). *Beauveria bassiana* has been found to be a benefit in controlling the rice weevil in stored grains especially when combined with other entomopathogens such as species of *Metarhizium* (Dal Bello et al., 2000). Although found to inhabit corn if it is injected into the plant,

Beauveria is not thought of as a plant pathogen (Bing and Lewis, 1992). *Beauveria bassiana* (strain GHA) produced by the MycoTech Corporation is in commercial production as a mycoinsecticide and will be explored here as a potential biological control in Florida citrus production.

The *Diaprepes* root weevil (*Diaprepes abbreviatus* L.) is a pest in Florida on citrus and other crops. It was first reported in the United States in a Florida nursery (Woodruff, 1964); now the weevil infests more than 50,000 ha of the 300,000 ha of citrus in production (Duncan et al., 2001). The weevil larvae feed on roots of the tree causing an entry court for fungal and other plant pathogens. It has been shown that *Phytophthora nicotinae* and *P. palmivora*, potentially lethal oomycetous organisms, can gain ingress into citrus roots where root integrity has been compromised by weevil damage (Rogers et al., 1996; Graham et al., 1997). The most severe decline in citrus has been linked to larval *Diaprepes* root damage and the phytoparasitic *Phytophthora palmivora* (Graham and Menge, 1999).

Sampling methods that have been published for estimating the relative abundance of larval *Diaprepes* in citrus are generally costly and can be damaging to trees in groves being sampled (Duncan and McCoy, 1996; Duncan et al., 1996). Several attempts have been made to produce an attractant for *Diaprepes abbreviatus* L., but none to date have been successfully marketed as a field attractant (Jones and Schroeder, 1984; Harari and Landolt, 1997). Above ground sampling with traps relies on the propensity of weevils to climb vertical surfaces rather than pheromone or food based chemical attractants. A modified Tedders trap has been used in citrus and proven valuable in estimating the relative abundance of adult root weevils in an infested area (Adair, 1994; Tedders and

Wood, 1994; Stansly et al., 1997; Prokopy and Wright, 1998; Mizell and Tedders, 1999; Duncan et al., 2001). Ecological studies have shown that there can be two peak periods for weevil emergence, in times of increased soil moisture, from April to mid June or late August to mid October (McCoy et al, 2003). A modification of this Tedders trap to infect weevils will be explored in the summary of this work with the hope that weevils passing through the trap will become infected with the conidia of *Beauveria* and be spread to other weevils through sexual contact or grooming.

Current biological control of the *Diaprepes* root weevil relies on the use of natural populations of entomopathogenic nematodes (Adair, 1994; Duncan and McCoy, 1996; Duncan et al., 1996; Duncan et al., 2001). Current chemical control of *Diaprepes abbreviatus* relies on timed applications of pesticides, but these control measures have yet to eliminate weevil populations (Bullock et al., 1988). With the growing concerns over the use of chemical pesticides, their costs and deleterious effects to our environment, it is important to explore the potential of enhancing biological control.

This study was initiated to examine the propensity of a readily available mycoinsecticide to infect adult *Diaprepes abbreviatus*. It is known that *B. bassiana* will infect adult and larval *D. abbreviatus* in nature (Futch and McCoy, 1992). In chapter 2, fungal mode of ingress and germination rates on different areas of adult *Diaprepes* integument was determined. This information could be used to identify target areas for conidial contact when a spore dissemination method is established. In chapter 3, *Diaprepes* sexual dimorphism was considered as a factor in internal fungal growth and development. External germination and penetration as well as internal fungal development and ramification were studied. Information gathered from chapters 2 and 3

was used in chapter 4 to determine the propensity of *B. bassiana* conidia to be transferred between same sex and intersexual contact. Chapter 5 presents a summary and conclusion for this study as well as possible future endeavors in this area.

CHAPTER 2
PROPENSITY OF *Beauveria bassiana* TO INFECT ADULT *Diaprepes abbreviatus*, ESTABLISHING ATTACHMENT AND GERMINATION

Introduction

Beauveria bassiana has long been identified as a disease-causing agent in insects (Steinhaus, 1949). Steinhaus provided an extensive history of the fungus. According to Ainsworth (1956), Agostino Bassi was one of the first people to experiment with insects infected with *Beauveria*. Bassi (1835) bred and inoculated silkworms (*Bombyx mori*) in an attempt to simulate muscardine disease, a disease characterized by insect death followed by a whitening of the exoskeleton. He devised many schemes to induce the disease, including several attempts that produced similar symptoms, but all of these failed at inducing disease in healthy insects. Bassi (1835) concluded that muscardine disease was caused by "a plant of the cryptogamic kind, a parasitic fungus."

Vuillemin (1912) was the first to propose the genus *Beauveria* in which he considered *Beauveria bassiana* as the type species. De Hoog (1972) has done the most definitive work on the genus *Beauveria* in which he was able to delimitate *Beauveria* from the genera *Isaria, Tritit achium* and *Acrodontium*. Scrutinized as a biological control agent of hypogeous insects, *Beauveria bassiana* has been found around the world and in many habitats (Ferron, 1981). This fungus regulates populations of different coleopterous insects of the genera *Diabrotica, Colaspis,* and *Maecolaspis* in soybean grown in Argentina and Brazil. In Brazil, the fungus achieves high prevalence in populations of

Aracanthus, an important pest of beans (Sosa-Gomez et al., 1994), and in France, England, and Morocco it can be found on Sitonia weevils, a major pest of cultivated Fabaceae (Poprawski et al., 1988). Isolates from the United States, Canada and China have also been collected and used in immunochemical characterization to identify a quality control program to ensure virulence of a strain (Tan and Ekramoddoullah, 1990). Although most *B. bassiana* isolates are morphologically indistinguishable, they vary in virulence. Biochemical characterization and large-scale production of these virulent isolates are important for successful biological control (Zhang and Tan, 1987). The conidia of *Beauveria* can be mass produced in quantities sufficient for use in microbial control, and can be produced on a semi-solid bran medium similar to that used in the commercial production of *Bacillus thuringiensis* (Soares et al., 1979). *Beauveria bassiana* (strain GHA) produced by the MycoTech Corporation is in commercial production as a mycoinsecticide.

The *Diaprepes* root weevil (*Diaprepes abbreviatus* L.) is a pest in Florida on citrus and other crops (Fig. 2-1). It was first reported in the United States in a Florida nursery (Woodruff, 1964); now the weevil infests more than 50,000 ha of the 300,000 ha of citrus in production (Duncan and McCoy, 2001). The weevil larvae feed on roots of trees creating an entry court for fungal and other plant pathogens. It has been shown that species of *Phytophthora*, potentially lethal plant pathogens, can gain ingress into citrus root systems where root integrity has been compromised by weevil damage (Rogers et al., 1996; Graham et al., 1997). Graham et al.(1997) found that in some soil types *Phytophthora palmivora* caused more damage to structural roots than the weevil larvae. Current control of the *Diaprepes* root weevil *(Diaprepes abbreviatus)* relies on timed

Figure 2-1. Adult *Diaprepes abbreviatus* female on citrus.

applications of chemical pesticides, but these control measures have yet to suppress weevil populations (Bullock et al., 1988). With the growing concerns in the use of chemical pesticides, their costs and deleterious effects to our environment, it is important that we explore the potential of enhancing biological control.

This study was initiated to examine the propensity of a formulated strain of *B. bassiana* to infect adult *Diaprepes abbreviatus*. It is known that *B. bassiana* will infect adult and larval *D. abbreviatus* in nature (Futch and McCoy, 1992). However, fungal modes of ingress and germination rates on different areas of adult *Diaprepes* integument have yet to be established. Humidity and temperature are necessary factors for the germination of *Beauveria bassiana* (Thomas and Blanford, 2003); however, neither humidity nor water alone is enough to stimulate germination of conidia (Hunt et al., 1984). The insect integument is known to have chemical compounds that affect the germination of *B. bassiana*. A lack of nutrients on sclerotized beetle cuticle is a limiting factor in fungal growth and development, including conidial germination (Hunt et al., 1984). Amino acids and glucosamine have been found on the larval cuticle of *Heliothis zea.* These fluctuate in levels during larval development but are always sufficient to trigger the germination of *B. bassiana* (Woods and Grula, 1984). Amines and peptides on the larval exoskeleton of *Heliothis zea* do not inhibit the germination of *B. bassiana* (Woods and Grula, 1984). Information gathered on the most vulnerable areas of insect integument will be considered in the future production of a spore dissemination method for the use of *Beauveria* in an integrated pest management system used to control levels of *Diaprepes abbreviatus.*

Materials and Methods

Mode of Ingress

Cages (Sho-Bowl, 64 oz. tub, ribbed dome lid, Ultra Pac, Rogers, Mn. 55374) with pin holes for ventilation in the lid were prepared that contained ~100g dry play sand. The sand and cages were sterilized under a UV hood for one hour. Surfaces were cleaned with a 10% Clorox® solution to avoid contamination of control insects.

Diaprepes abbreviatus were reared at the Florida Department of Agriculture facility in Gainesville, Florida, using Beavers' (1982) methods for rearing on an artificial diet. A dry commercially produced wettable powder formulation of *B. bassiana* conidia produced by the MycoTech Corporation (strain GHA, Mycotrol ES, Emerald BioAgriculture, Butte, MT, 59702) was used as an inoculum. This strain was previously used by Furlong and Groden (2003) in their study of the alfalfa weevil, *Hypera posica*. Weevils were separated by sex (Harari and Landolt, 1997) then placed in batches into clean plastic sandwich bags containing 0.05g powder per insect. Bags with insects and powder were shaken for thirty seconds. This method of conidial delivery was similar to that used by Hedlund and Pass (1968). Insects were then transferred to holding cages, based on sex and treatment, with two organically grown baby carrots and a cotton ball saturated with sterilized water. Control groups were agitated in a clean plastic bag to simulate the possibility of mechanical damage insects might have incurred during treatment. Insects were maintained in an incubator with a 14-10 hour light cycle, a relative humidity of 85-90%, and a temperature range between 22 and 24°C.

Thirty minutes after treatment, 5 insects of each sex were removed from the holding containers and the live insects were pinned through the right elytra with a number 2 nylon headed insect pin. Insects were then dipped into a collodion solution (Collodion

Flexible USP, Alcohol 22%, Ether 67%, Pyroxylin 4.75%, Camphor 2% and Castor Oil 3%, HUMCO, Texarkana, Texas 75501), and allowed to stand at room temperature until the layer was solidified but not hardened (approximately 45 minutes). The solidified collodion film was then removed from the insect cuticle with jeweler's forceps (Miltex, size 5). This process removes any loose or attached conidia and some insect cuticular components (Delp, 1954; Kimbrough, 1963). Glass slides (3 X 1 inch, Sargent-Welch Scientific Company, Skokie, Illinois 60076) were prepared using lactophenol cotton blue stain. Lactophenol cotton blue stain was made by mixing 20 g phenol crystals (dissolved by gentle heating), 20 ml lactic acid, 40 ml glycerol, 20 ml distilled water, and 0.05 g cotton blue (=aniline blue) (Shurtleff and Averre, 1997). Peels were made concentrating on the abdominal sterna and related inter-segmental membranes, and from the elytra to the beak including the pronotum, eyes, and antennae.

Each peel was placed with the exoskeleton contact side facing up to reduce interference from the collodion when visualizing the peel with the compound light microscope. A drop of lactophenol cotton blue stain was placed on the peels of collodion. After 5 minutes excess stain was wicked off of the peels with absorbent tissue. The slides were then sealed using a few drops of Cytoseal™ 60 (Stephens Scientific, Division of Richard-Allan Scientific, Kalamazoo, MI 49007-3538) and a 50 x 24mm cover glass (Thickness 1). To ensure thin slides and remove air bubbles, slides were placed on a slide warmer for six hours at 50°C with a 15g lead weight on the cover glass. Slides were removed from the slide warmer and allowed to cool for six hours before the weight was removed from the cover glass. Collodion dipping and the preparation of epidermal peels were repeated every six hours with fresh insects from the treatment cohort until 30 hours

past the initial inoculations. Conidial density, germination rate and growth, including the formation of germ tubes and points of fungal ingress were observed and photographed using a Nikon 990 digital camera attached to a light microscope. Dorsal (Fig. 2-2) and ventral (Fig. 2-3) photographs of weevils were labeled for rapid identification of areas.

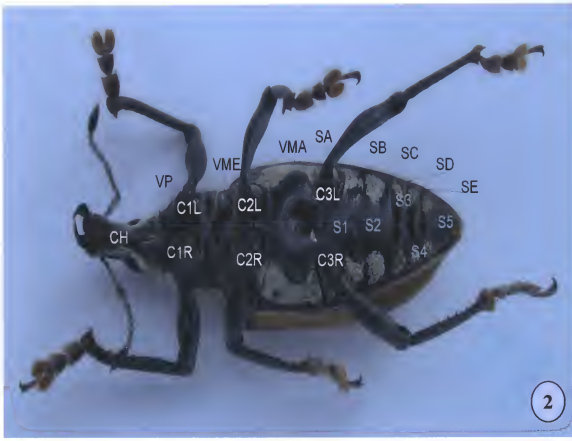


Figure 2-2. Ventral map of *Diaprepes abbreviatus* female.

CH- chin
VP- ventral pronotum
C1L- coxa 1 left
C1R- coxa 1 right
VME- ventral mesosternum
C2L- coxa 2 left
C2R- coxa 2 right
VMA- ventral metasternum
C3L- coxa 3 left
C3R- coxa 3 right
S1-5- sterna
SA-E- inter sterna membranes

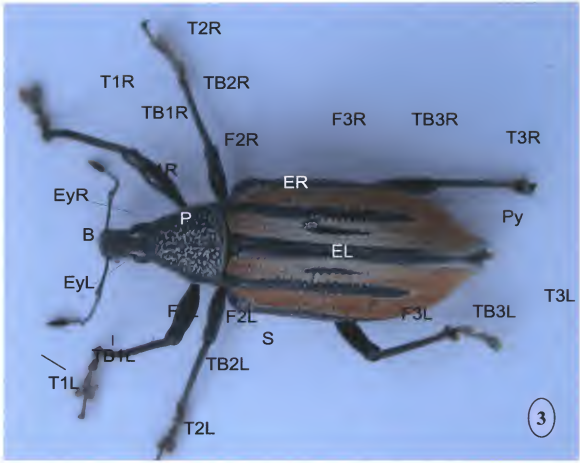


Figure 2-3. Dorsal map of *Diaprepes abbreviatus* female.

R- right
L- left
Ey- eye
B- beak
P- pronotum
E- elytra
F- femur
TB- tibia
T- tarsus
S- scutellum
Py- pygidium

Conidial Viability and Weevil Contact

A simple procedure was used to determine the number of viable conidia on each treated insect. Insects were treated with 0.05g dry commercially produced wettable powder of *B. bassiana* per insect using the previously described plastic bag procedure. The weevils were then placed in a holding container for three hours, during this three hour time frame, any loose conidia were removed by weevil contact or agitation. The weevils were then removed from the holding container and placed individually in 100ml glass beakers. One ml of de-ionized water was added to the vial containing the weevil. The vial was then vigorously shaken for 1 minute. The water from each weevil was poured onto potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, 48232-7058). Forty grams of PDA were mixed with 1 l (1,000ml) of water in an Erlenmeyer flask and sterilized for 30 minutes at 15 PSI. Agar was poured in Fisher disposable Petri dishes (100x15MM) and allowed to cool before use. After conidia were placed on the surface of the agar, the Petri dishes were sealed with Parafilm M Lab Wrapping Film and placed inverted in an incubator at room temperature for 48 hours. Insects were treated with the powder then immediately washed. After incubation, the plates were examined for signs of fungal growth. Colonies forming on the plates were counted on a grid system under a dissecting microscope at 40 x. Data was analyzed using Excel (Microsoft Corp, 1997).

Results

Spores on the surface of the weevil were not evenly distributed 30 minutes post inoculation. Spore density on weevil surfaces could not be determined using the collodion method. Spore density on the weevils' surface was determined to be 15,815 ±

479 spores initially after inoculation. Spore load on the surface of the weevils was reduced 5,782 ± 136 spores after three hours. Thirty minutes post inoculation, conidia were found in clusters around ommatidia-EY (Fig. 2-4), seta-VP (Fig. 2-5 and 2-7), in depressions in the integument, ventral pronotum-VP (Fig. 2-6), and in the grooves of scales located along the ventral surfaces-S (Fig. 2-8). At six hours weevils were checked for signs of spore enlargement or germination. No evidence was found to suggest spore growth at that time. At 12 hours, peels were similar to those taken at 30 minutes and six hours post inoculation. Spores were found on plumose seta (Fig. 2-9), around the base of seta (Fig. 2-10) and in clusters around ommatidia-EY (Fig. 2-11). Spores were abundant in pits of pronotum and in the grooves between the ommatidia of both eyes. Debris from insect rearing media around edges of peels could account for spores clustering in those areas. Spores began to enlarge at 12 hours from 1.3 μm to 2.9 μm. Measurements were taken for spore length not width. Germination observed at 18 hours was 24 ± 3% on abdominal sections-S (Fig 2-12-14, 2-16), 10 ± 3% on the pronotum-P and beak-B (Fig 2-15), 35 ± 3% on the eyes-EY, and 9 ± 2% on scales on abdominal sections-S. Enlarged conidia were seen on the elytra-E but had not begun to germinate at 18 hours (Fig. 2-17). Germination observed at 30 hours was 58 ± 3% on the pronotum-P (Fig. 2-18), 74 ± 3% on the eyes-EY (Fig. 2-19-22), 65 ± 3% on abdominal sections-S (Fig. 2-23), and 44 ± 3% on scales on abdominal sections-S. Conidia 30 hours post inoculation were found germinating on the ovipositor-Py (Fig. 2-24), in clusters on the edge of sternal segments-S (Fig. 2-25-26), on the ventral metasternum- VMA near SA (Fig. 2-27), and among scales of sterna-S (Fig. 2-28). At 30 hours germination was seen on both of the elytra-E at a rate of 7 ± 3% (Fig. 2-29).

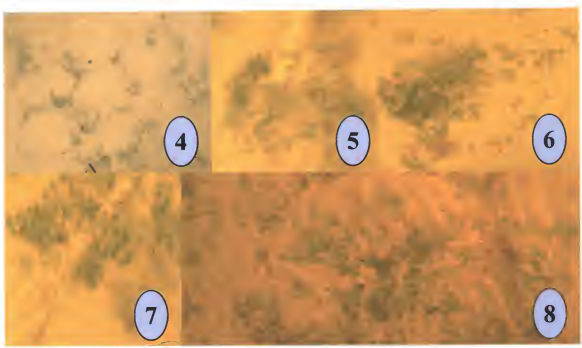


Figure 2-4-2-8. *Beauveria bassiana* conidia on *Diaprepes abbreviatus* exoskeleton 30 minutes post inoculation.

2-4. Conidia in clusters around ommatidia of right eye.
2-5. Conidia in clusters around hair on chin.
2-6. Conidia in clusters on pronotum-P.
2-7. Conidia in clusters around hair on coax 3 left.
2-8. Conidia in grooves of scales of ventral metasternum.

Figure 2-9-2-11. *Beauveria bassiana* conidia on *Diaprepes abbreviatus* exoskeleton 12 hours post inoculation.

2-9. Conidia in clusters around hair on beak.

2-10. Conidia in clusters around hair on sterna 2.

2-11. Conidia in clusters around ommatidia of left eye.

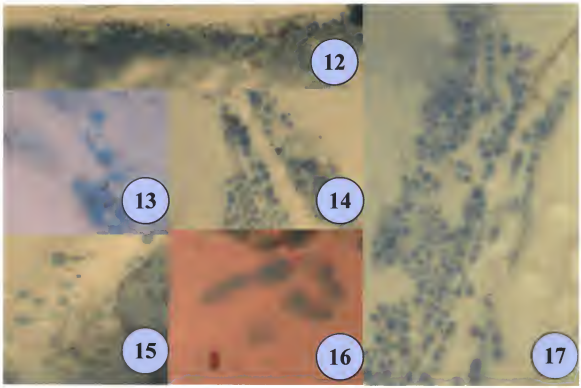


Figure 2-12-2-17. *Beauveria bassiana* conidia on *Diaprepes abbreviatus* exoskeleton 18 hours post inoculation.

2-12. Conidia in grove of sterna E.
2-13. Conidia germinating on sterna 5.
2-14. Conidia germinating around hair of sterna 2.
2-15. Conidia germinating on beak.
2-16. Conidia germinating on the ventral metasternum near SA.
2-17. Conidia in clusters along ER near S and P.

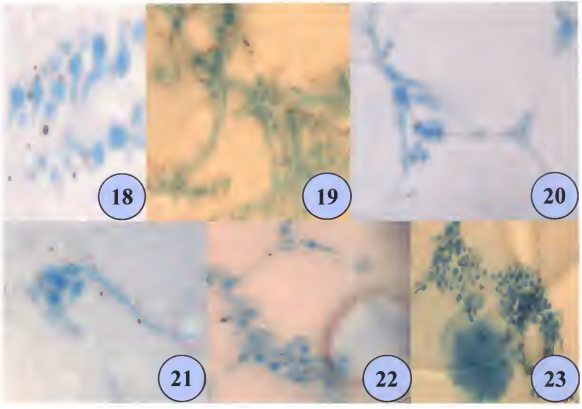


Figure 2-18-2-23. *Beauveria bassiana* conidia on *Diaprepes abbreviatus* exoskeleton 30 hours post inoculation.

2-18. Conidia germinating on pronotum
2-19. Conidia germinating on ommatidia of left eye.
2-20. Conidia germinating on edge of eye.
2-21. Conidia germinating on beak near eye.
2-22. Conidia germinating on ommatidia of right eye.
2-23. Conidia germinating around hair on sterna 1.

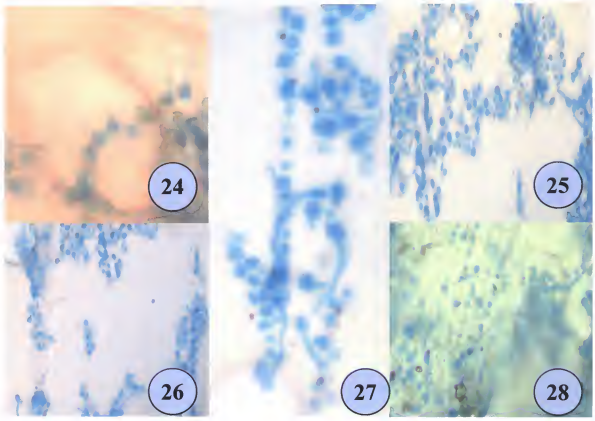


Figure 2-24-2-28. *Beauveria bassiana* conidia on *Diaprepes abbreviatus* exoskeleton 30 hours post inoculation.

2-24. Conidia germinating on ovipositor.
2-25. Conidia germinating in cluster on edge of sterna 4.
2-26. Conidia germinating on edge of sterna 3.
2-27. Conidia germinating on VMA near SA.
2-28. Conidia in clusters germinating among scales of sterna 1.

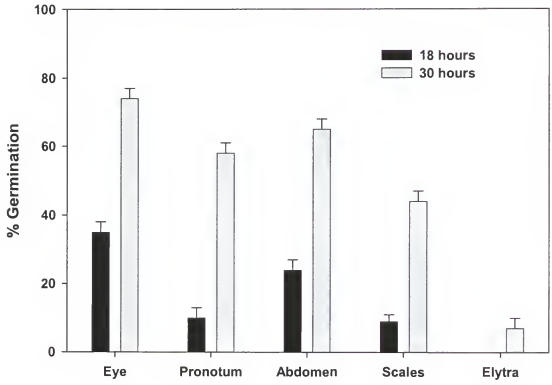


Figure 2-29. Percentage of *Beauveria bassiana* conidia germinating on different areas of *Diaprepes abbreviatus* exoskeleton at 18 and 30 hours post inoculation.

Discussion

Diaprepes weevils were infected with a dry, commercially produced wettable powder of *B. bassiana* conidia, strain GHA, a strain previously used in studies of Colorado potato beetle larvae by Furlong and Groden (2003). Conidial lengths of *B. bassiana* isolates tested by Sosa-Gomez et al. (1994) showed lengths varying among isolates from 0.95 to 3.41 μm, these dimensions agree with those collected at the time of inoculation. The development of *B. bassiana* on adult and larval *D. abbreviatus* is similar to other entomopathogenic deuteromycetes, including *Nomuraea* (Boucias and Pendland, 1982).

Essentially nothing was known as to how spores of *B. bassiana* would cling and aggregate on the surface of the *Diaprepes* root weevil after inoculation. Data from this research shows that conidia tend to condense in intersegmental areas and around hairs and the base of appendages. Natural grooves in scales also fill with spores. There has been little definitive data as to the timetable of spore germination once spores make contact with adult *D. abbreviatus* integument. We have learned that spores germinate at different rates on different areas of the weevils' exoskeleton. Several authors have studied why *B. bassiana* spores germinate when they come in contact with insect cuticle. Reasons for germination and penetration include compounds on insect integument, for example amino acids, glucosamine, amines and peptides on the larval exoskeleton of *Heliothis zea* did not inhibit the germination of *B. bassiana* (Woods and Grula, 1984). However, the lack of nutrients on sclerotized beetle cuticle is a limiting factor in fungal growth and development (Hunt et al., 1984). Results obtained from this study indicate that germination is limited on the heavily sclerotized elytra (Fig. 2-29). By observing germination rates on various regions we are aware of where the largest numbers of spores

will germinate most readily on *Diaprepes* integument as well as conidial viability in a dry commercially available wettable powder. Data generated in this phase of research will enable one to project the likely source of direct fungal entry and provide researchers with the information they need to produce an effective conidial delivery system for effective *Diaprepes* root weevil control.

CHAPTER 3
Beauveria bassiana INTERNAL GROWTH AND DEVELOPMENT IN ADULT *Diaprepes abbreviatus*

Introduction

As was noted in the previous chapters, *Diaprepes abbreviatus* (Fig 2-1) is an important pest on citrus and other crops in Florida. Known as the *Diaprepes* root weevil, it causes major damage in grove trees and in nurseries where rootstocks are developed. Not only does the weevil damage the roots, it makes the trees susceptible to soil-borne fungi and other pathogens (Chapters 1-2). According to Schneider (2000) the weevil cost growers more than 71 million dollars per year in fruit and tree loss combined with the cost to control the weevils' spread.

Currently, there are no known ways to eradicate *Diaprepes abbreviatus*, although it can be chemically controlled to a limited degree. Chemical control, however, is costly and dangerous to our environment (Carson, 1962) and no rootstock appears to be resistant to larval feeding (Futch and McCoy, 1992). As farmers look for a more environmentally sound, safer and more selective approach to crop protection, biopesticides come of value in integrated pest management (Hall and Menn, 1999; Menn, 1996).

The use of the naturally occurring entomopathogenic fungus *Beauveria bassiana* as a biopesticide or mycoinsectiside on various pests has a long standing history (e.g., Anderson and Roberts, 1983; Anderson et al., 1989; Fargues et al., 1983; Furlong and Groden, 2003; Hajek et al., 1987; Quintela and McCoy, 1997, 1998; Tanada and Kaya,

1986; Wraight and Carruthers, 1999). In previous work (chapter 2), mode of ingress and germination rates of *Beauveria bassiana* on different areas of adult *Diaprepes* integument were established. Once penetration of the exoskeleton has occurred, *B. bassiana,* like all entomopathogens, has a host of insect immune responses to overcome (Gupta, 1979).

Insects have a wide diversity of hemocytes in their hemolymph responsible for the control of foreign invaders and artificially introduced objects (Brehelin et al., 1975). The ultrastructure of these hemocytes has been studied at great lengths for many insect orders. There are many controversies because certain hemocytes undergo rapid transformation soon after collection which can lead to incorrect descriptions (Brehelin et al., 1978). Little is known about the hemocyte activity of Coleoptera; this area has recently been explored but has not yet been established. Ultrastructure and functional morphology of hemocytes of *Cetonischema aeruginosa* larvae (Coleoptera, Scarabaeidae) have shown the involvement of both granulocytes and oenocytoids in *in vivo* phagocytosis (Giulianini et al., 2003). This study was conducted to identify internal fungal development of *Beauveria bassiana* undergoes initial penetration of *Diaprepes* exoskeleton and what, if any, hemocyte activity occurs.

Materials and Methods

Hematology

Adult *Diaprepes abbreviatus* were reared at the Florida Department of Agriculture facility in Gainesville, Florida, using Beavers' (1982) methods for rearing on an artificial diet. Weevils were separated by sex using the method of Harari and Landolt (1997) prior to treatment. Cages were prepared and weevils were inoculated with a dry commercially produced wettable powder of *B. bassiana* conidia as described in chapter 2, using 0.05g

powder per insect. Insects were then transferred to holding cages, based on sex and treatment, along with a cotton ball saturated with sterilized water. Insects were maintained in an incubator with a 14-10 light cycle, a relative humidity of 85-90%, and a temperature range between 22ºC and 24ºC.

Hemolymph was removed from the abdomen with a 30 gauge hypodermic needle and then placed on glass slides (3 X 1 inch, Sargent-Welch Scientific Company, Skokie, Illinois 60076). A 50 x 24mm cover glass (Thickness 1) was then placed on top of the hemolymph sample with no other mounting media (Pendland et al., 1993). A second slide was prepared from the same insect by cutting through the femur near the tibia on the right foreleg with a pair of dissecting scissors. Hemolymph from this wound was also extracted and placed on a glass slide. Slide mounts of hemolymph were made from uninjured male and female insects from the same treatment cohort every 24 hours or until hemolymph could no longer be removed or insect death occurred. Slides of hemolymph were observed under phase-interference microscopy and photographed within 15 minutes of preparation using light microscopy. Hemolymph from healthy male and female insects was prepared as controls and photographed using light microscopy.

Internal Development and Ramification

Insects prepared as above and maintained in the same conditions were treated with powder of *B. bassiana* conidia as described in chapter 2, using 0.05g powder per insect. These insects were prepared for thin sectioning by placing them in 16 dram glass vials with enough 40% mucilage to cover them. The specimens were placed in a refrigerator for 24 hours then frozen in mucilage until sectioning was performed. Specimens were mounted in 40% mucilage then frozen with a cryostat at -20ºC (Kimbrough and Lenz,

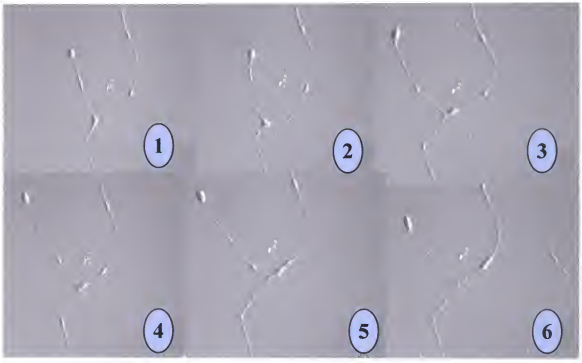
1982; Bezerra and Kimbrough, 1975). Insects were mounted without legs and elytra in two pieces; the head and thorax were mounted together and the abdomen was mounted on a separate stub. Frozen sections approximately 14μm thick were transferred to glass slides and stained with lactophenol-cotton blue, sealed with clear nail polish and then observed and photographed using light microscopy. Five male and five female insects were prepared every 24 hours from the time of inoculation to the time when external hyphal growth was observed on the exoskeleton.

Results

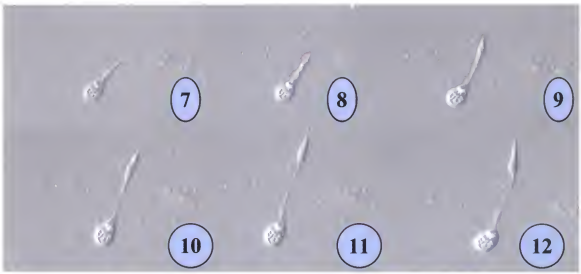
Hemolymph from healthy adults showed *Diaprepes abbreviatus* hemocyte activity (Fig. 3-1 to 3-6). Two days after the initial inoculation, hemocytes became less filamentous (Fig. 3-7 to 3-12). Hemolymph was extracted from different areas of male and female insects. After 24 hours (Fig. 3-7 to 3-12) and 48 hours (Fig. 3-19 to 3-24) there was no discernable difference in hemocyte shape or activity based on body area or sex of the weevils. At 72 hours post inoculation, female weevils appeared free of hyphal bodies and had similar hemocytes to those found at 24 and 48 hours. Male weevil hemocytes collected from the abdomen were similar to those found at 24 and 48 hours. At 72 hours the appearance of hemocytes engulfing hyphal bodies and free floating hyphal bodies could be seen in hemolymph collected from the legs of males. At 96 hours post inoculation (Fig. 3-31-36) hemocytes and hyphal bodies could be seen in male and female insects. At this time hemocytes and branching septate hyphae could be found in female abdominal hemolymph (Fig. 3-31). Hemolymph from female legs was found to have hemocytes, hyphal bodies and budding hyphal bodies (Fig. 3-32,33). Male and female hemolymph from all areas still had active hemocytes at this time (Fig. 3-34).

Hemolymph from males did not show any branching hyphal bodies at this time but did show septate and budding hyphal bodies from the legs (Fig. 3-35-36).

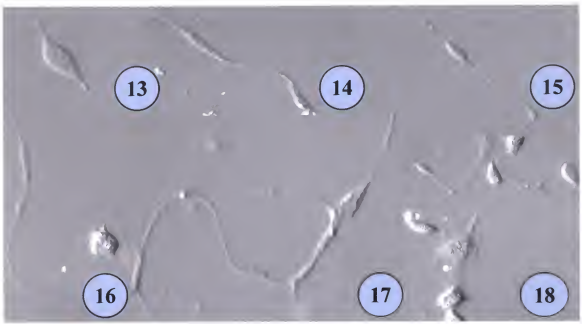
Branching and budding hyphal bodies became more prolific in female hemolymph from legs and abdomen 120 hours post inoculation (Fig. 3-37-39). Male hemolymph contained numerous hyphal bodies some of which could be found being encircled by hemocytes (Fig. 3-40) while others were seen budding (Fig. 3-41-42). Although many male insects were still alive at 144 hours post inoculation, it was impossible to remove hemolymph from their legs or abdomen at this time. Female weevils had hemocytes, free hyphal bodies, and branching septate hyphae in their abdominal hemolymph at 144 hours post inoculation (Fig. 3-43). At this time female legs were found to contain hemocytes, free hyphal bodies, branching septate hyphae, and budding hyphal bodies (Fig. 3-44-48). At 168 hours post inoculation it became impossible to extract hemolymph from living female insects. Internal hyphal development continued after it became impossible to remove hemolymph. Two to three days after weevil death sporulation occurred.



Figures 3-1-3-6. Progression of hemocyte movement in an untreated adult *Diaprepes abbreviatus*, photos in series taken every 20 seconds.



Figures 3-7-3-12. Progression of hemocyte movement in an infected adult *Diaprepes abbreviatus*, 48 hours post inoculation with *Beauveria bassiana* conidia. Photos in series taken every 20 seconds.



Figures 3-13-3-18. Hemocytes in *Diaprepes abbreviatus* hemolymph 24 hours post inoculation with *Beauveria bassiana* conidia.

3-13. Hemolymph from female abdomen.
3-14. Hemolymph from female abdomen.
3-15. Hemolymph from female leg.
3-16. Hemolymph from male leg.
3-17. Hemolymph from male leg.
3-18. Hemolymph from male leg.

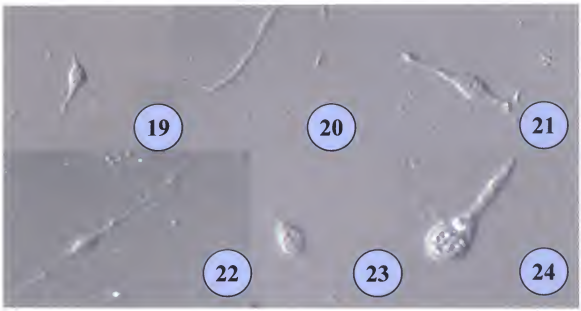


Figure 3-19-3-24. Hemocytes in *Diaprepes abbreviatus* hemolymph 48 hours post inoculation with *Beauveria bassiana* conidia.

3-19. Hemolymph from female abdomen.
3-20. Hemolymph from female abdomen.
3-21. Hemolymph from female leg.
3-22. Hemolymph from male abdomen.
3-23. Hemolymph from male leg.
3-24. Hemolymph from male leg.

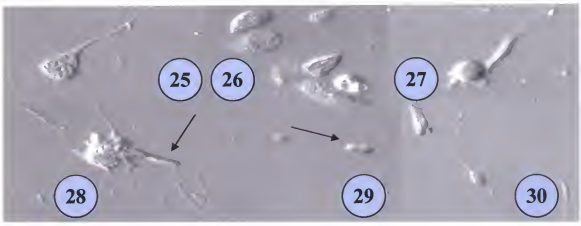


Figure 3-25-3-30. Hemocytes and hyphal bodies in *Diaprepes abbreviatus* hemolymph 72 hours post inoculation with *Beauveria bassiana* conidia.

3-25. Hemolymph with hemocyte from female abdomen.
3-26. Hemolymph with hemocyte from female leg.
3-27. Hemolymph with hemocyte from male abdomen.
3-28. Hemolymph from male leg, hyphal body being engulfed by hemocyte.
3-29. Hemolymph with hemocyte from male leg with free hyphal body.
3-30. Hemolymph with hemocyte from male leg.

Figure 3-31-3-36. Hemocytes and hyphal bodies in *Diaprepes abbreviatus* hemolymph 96 hours post inoculation with *Beauveria bassiana* conidia.

3-31. Hemolymph with hemocytes and branching septate hyphae from female abdomen.
3-32. Hemolymph with hemocytes hyphal bodies and a budding hyphal body from female leg.
3-33. Hemolymph with hemocytes and septate hyphal body from female leg.
3-34. Hemolymph with hemocytes from male abdomen.
3-35. Hemolymph with hemocytes from male leg with free hyphal body
3-36. Hemolymph with hemocytes and dividing hyphal body from male leg.

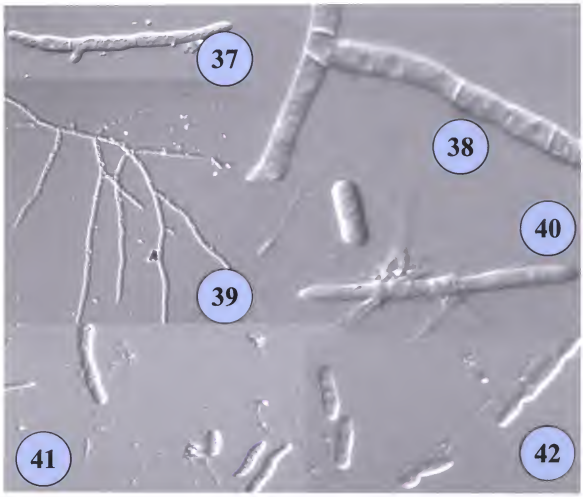


Figure 3-37-3-42. Hemocytes and hyphal bodies in *Diaprepes abbreviatus* hemolymph 120 hours post inoculation with *Beauveria bassiana* conidia.

3-37. Hemolymph with hemocytes and a budding hyphal body from female abdomen.

3-38. Hemolymph with septate hyphal branching from female leg.

3-39. Hemolymph with hyphal body and branching septate hyphae from female abdomen.

3-40. Hemolymph with hemocytes encircling hyphal body from male leg.

3-41. Hemolymph with hemocytes from male leg with free hyphal bodies.

3-42. Hemolymph with hemocytes and dividing hyphal bodies from male leg.

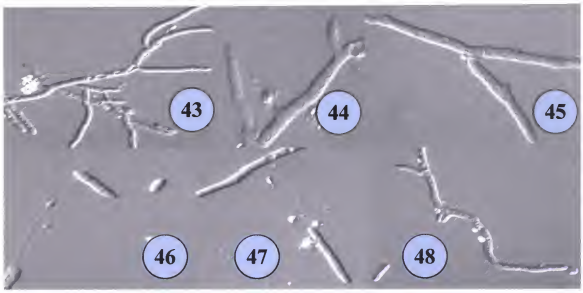


Figure 3-43-3-48. Hemocytes and hyphal bodies in *Diaprepes abbreviatus* hemolymph 144 hours post inoculation with *Beauveria bassiana* conidia.

3-43. Hemolymph with hemocytes, free hyphal bodies and branching septate hyphae from female abdomen.

3-44. Hemolymph with hemocytes and a branching budding hyphal body from female leg.

3-45. Hemolymph with a septate hyphal body from female leg.

3-46. Hemolymph with hemocytes from female leg with 2 small hyphal bodies.

3-47. Hemolymph with hemocytes from female leg with hyphal body and hyphae.

3-48. Hemolymph with hemocytes, dividing hyphal body and free hyphal body from female leg.

Discussion

Initial examinations of healthy adult hemolymph showed *Diaprepes abbreviatus* hemocyte activity (Fig. 3-1 to 3-6). Movement of hemocytes in samples from infected weevils showed similar movement; however, hemocyte numbers were reduced in later samples. These hemocytes are similar to plasmatocytes as described by several authors (Brehélin et al., 1978; Giulianini et al., 2003; Gupta, 1979). Giulianini et al. (2003) were the first to describe plasmatocytes in Coleoptera as "the great number of highly heterogeneous membrane-enclosed vesicles and infolded regions of the plasma membrane, as well as their capacity to become very elongated after activation." Hemocytes observed from *Diaprepes* adults fit the description of plasmatocytes as described by Giulianini et al. (Fig. 3-1-12).

Hemocyte movement and activity were interrupted by staining the hemolymph prior to microscopy. Hemolymph directly applied to a slide was observed and photographed within 15 minutes for this study; however, hemocytes remained active for more than 24 hours. In chapter 2 it was shown that conidia of *Beauveria bassiana* germinated on adult *Diaprepes abbreviatus* 18 hours post inoculation. Hyphal bodies became evident in the hemolymph 72 hours post inoculation. The discrepancy between penetration time and observation of hyphal bodies could be due to the encapsulation of hyphal bodies by hemocyte activity (Fig. 3-27). As hyphal body development continued, it became increasingly difficult to extract hemolymph from the living insects. Over time the size of hyphal bodies increased and could have blocked blood flow or plugged the extraction needle. This could account for the inability to extract hemolymph from male

and female insects that were still alive after 144 and 168 hours respectively. Internal growth of hyphae continued until the body cavity of the insect was consumed.

CHAPTER 4
HORIZONTAL TRANSMISSION OF *Beauveria bassiana* BY ADULT *Diaprepes abbreviatus* AND SUBSEQUENT MORTALITY RATE

Introduction

To understand male and female behavior in *Diaprepes abbreviatus,* several scientists have studied their interactions (Harari and Brockmann, 1999). It was noted that female on female mounting for a short time increased the females chance of mating with a larger male. They also noted that males will also mount other males. For this reason, transmission of *Beauveria bassiana* conidia both inter-sexually and intra-sexually was tested.

Previous research (chapters 2 and 3) on the effect of *Beauveria bassiana* on *Diaprepes abbreviatus* has established that insects are more susceptible to fungal ingress on thin areas of cuticle and that internal ramification of the fungus is not impeded greatly by insect hemocyte activity. The purpose of this study was to establish when male and female mortality occurred. Direct observation of the possibility of horizontal transmission (between individuals) of *Beauveria bassiana* from weevil contact could open a new venue for the use of this mycoinsecticide, using a commercial source of *B. bassiana* conidia.

Materials and Methods

Mortality Rates

As alluded to in previous chapters, *Diaprepes abbreviatus* were reared at the Florida Department of Agriculture facility in Gainesville, Florida, using Beavers' (1982) methods for rearing on artificial diet. Insects were separated by sex (Harari and Landolt, 1997), treated with 0.05g dry conidia of *Beauveria bassiana* and caged as in chapter 2. Insects were maintained in an incubator with a 14-10 light cycle, a relative humidity of 85-90%, and a temperature range between 22 and 24°C. Weevils were maintained with two organic baby carrots and a cotton ball saturated with sterilized water. On a daily basis the old carrots and cotton were replaced with fresh carrots and moist cotton. Cages were checked every 24 hours for mortality. Dead insects were collected and removed daily. They were maintained until sporulation occurred and *Beauveria* conidia were recovered.

Horizontal Transmission

Adult *Diaprepes abbreviatus* were separated by sex. By mating with larger males, females may benefit by acquiring 'good genes' or a greater nutrient source as materials are transferred in the sperm package from males to females (Harari et al., 1999). Therefore, weevils of similar size were grouped together. One male weevil was allowed to walk through a dry commercial wettable powder of *B. bassiana* conidia produced by the MycoTech Corporation (strain GHA, Mycotrol ES, Emerald BioAgriculture, Butte, MT, 59702). This weevil was then placed in a holding container for 24 hours with an untreated female from the same cohort. The female was removed from the holding container and placed in the one ounce diet cup in which she was reared as described by Beavers (1982). At this time the treated male was held in the mounting position for one minute on a male from the same cohort, the newly touched male was placed back in the

container in which it was reared. This mock mounting was repeated with a female. Each day two males and two females were mounted with the initial treated male. Females and males were held until sporulation occurred and *Beauveria* was confirmed as the cause of death. This cycle was repeated for three days. This experiment was repeated using female insects as the initial infected insect. At this time the treated female was held in the mounting position for one minute on a female from the same cohort then mounted on a male. Containers were checked every 24 hours for mortality. Dead insects were maintained until sporulation occurred and *Beauveria* conidia were recovered using a common tape mount method (Shurtleff and Averre, 1997) and a drop of lactophenol cotton blue stain. Control insects were mounted with one another and held in the same manner as the treatment group. Mortality was compared using contingency χ^2 tests (Zar, 1974), Excel and multiple regression (General Linear Model) in SAS (Microsoft Corp, 1997; SAS Institute Inc., 2001).

Results

Diaprepes abbreviatus treated with 0.05g dry conidia of *Beauveria bassiana* experienced 100% mortality (Fig 4-1). Control insects exhibited no symptoms and experienced no disease related mortality. Infected male and female mortality began 6 days post inoculation. The longest lived diseased male survived 21 days post inoculation compared with the longest lived female which survived 17 days post inoculation. In the time between inoculation and death, the greatest numbers of males and females died 9-13 days post inoculation. There was no significant difference between the rate at which males died compared to females (Fig 4-1).

To confirm that mortality was caused by *Beauveria bassiana,* dead insects were observed for sporulation (Fig. 4-2). *Diaprepes abbreviatus* showing signs of *Beauveria bassiana* development were collected and checked for conidia and conidiophores on antennae and chin-CH (Fig. 4-3), at junction of femur-F and tibia-TB (Fig. 4-4), mouthparts-B and chin-CH (Fig. 4-5), and on antennal segments (Fig. 4-6). Insects were confirmed dead by means of *Beauveria bassiana* if conidia and conidiophores were found.

Overall mortality in both males and females mock mated to inoculum carrying adults was significantly higher than mortality in insects mock mated to uninfeted individuals (χ^2= 57.4; df=1; p<0.001). One false mortality after exposure to uninfected insects was added for statistical convenience.

After 24 hours females and males that were allowed to walk through dry conidia were mock mated for one minute to another female, then to a male. This was repeated for a total of two intersexual and two intrasexual contacts, and process was repeated every 24 hours for three days. There was no significant difference between male and female ability to transmit enough *Beauveria bassiana* conidia to cause mortality in another insect (χ^2= 0.03; df=1; p>0.98). Neither was male and female susceptibility to fungal infection in mock mating significantly different (χ^2=0.0047; df=1; p< 0.95).

The number of spores transferred to an uninfected insect may influence the virulence of the resulting infection. If sequential contacts between an infected adult and an uninfected receiving weevil resulted in a decreased number of available spores, then the position within a series of interactions could influence the virulence of resulting infections. This might be reflected in differences in post contact longevity. However, a

General Linear Model that attempted to explain the variance in the post exposure longevity of infected weevils on the basis of sex of the transmitting individual, the sex of the receiving individual and its position within a series of 12 mock mating contacts failed to find a significant relationship between life span and any of the variables or their interactions ($F=1.22$; 5; total df's 69; $p=0.31$; $r^2=0.09$).

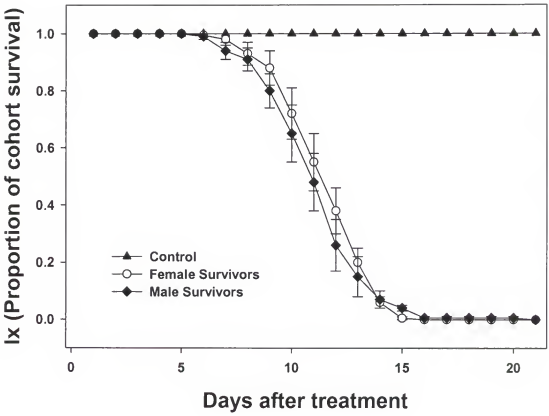


Figure 4-1. Proportion of *Diaprepes abbreviatus* adults surviving treatment with 0.05g dry *Beauveria bassiana* conidia.

Figure 4-2. *Diaprepes abbreviatus* showing evidence of *Beauveria bassiana* development.

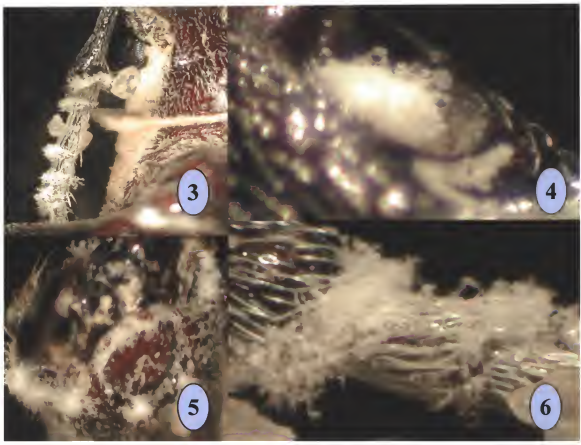


Figure 4-3-4-6. *Diaprepes abbreviatus* 312 hours post inoculation with *Beauveria bassiana* conidia.
4-3. Sporulation on antennae and neck.
4-4. Sporulation at junction of femur and tibia.
4-5. Sporulation on mouthparts and chin.
4-6. Sporulation on antennal segments.

Discussion

Weevil life span has been measured at 147 ± 17.1 days for females and 135 ± 21.5 days for males (Beavers, 1982). Weevil death from contact with *B. bassiana* inoculum ranged from 6 to 21 days post contact, the majority dying between 7 and 10 days. It was previously noted that females would remain mounted with other females up to 17 minutes (mean 9.6 min) in the laboratory, whereas males would remain mounted for more then 10 hours (Harari and Brockmann, 1999). In later studies, estimates of male mounting time increased to more than 16 hours in copula. It was shown that females who are not mated until dusk have a good chance of coming in contact and mating with another male (Harari et al., 2003). Multiple sexual partners could increase the probability of horizontal transmission.

Iridovirus transmission has been shown vertically (transovarially) and horizontally in the *Diaprepes* root weevil (Hunter et al., 2003). Unlike the viral transmission, weevil death occurred prior to reproductive maturity, so vertical transmission of *B. bassiana* could not be established. With horizontal transmission it was shown that weevils that came in contact with *B. bassiana* were capable of spreading disease to other weevils they came in contact with. Gender played no role in transmission either inter and intra sexually. Wolcott (1936) reported that there was a preoviposition period of three to seven days from the time that female weevils emerged from the soil to the time they began to lay eggs. Later it was shown that the preoviposition stage was much longer then previously reported and could last as long as 22 ± 3 days (Beavers, 1982). If weevils were to contact *B. bassiana* conidia as they emerge from the soil and die prior to reaching reproductive maturity, the effect of the fungus on control might be particularly

efficacious. It was shown that the day of infestation did not have any impact on weevils ability to transmit enough conidia to cause mortality. These data show that horizontal transmission of *B. bassiana* can be very instrumental in helping to reduce the number of eggs laid by adult females. This could have a great impact on weevil populations in the field.

CHAPTER 5
SUMMARY AND CONCLUSIONS

From Woodruff's first report of the *Diaprepes* root weevil's entry into Florida in 1964, it has become a serious pest in all areas of Florida that produce citrus (McCoy and Simpson, 1994). With an estimated economic impact of 70 million dollars a year it is one of the most serious threats to the Florida Citrus industry (Schneider, 2000). Attempts to control the weevil include chemical as well as biological control. The use of entomopathogenic fungi as explored in the last three chapters appears to have promise as a new form of biological control for *Diaprepes* root weevil adults. Timing of fungal application will be critical in adult control. *Beauveria bassiana* occurs as an entomopathogen in Florida (Kish et al., 1974). To avoid contacting beneficial insects with fungal inocula, spray application of *B. bassiana* would not be recommended. Some concerns about the use of *B. bassiana* have arisen from the report that beauvericin isolated from mycelia was toxic to brine shrimp and mosquito larvae (Hamill et al., 1969).

The Show-bowl cages proved very successful for maintaining the weevils and avoiding contamination of controls that were housed in the same incubator as the treatments. Very consistent results were obtained with the conidia produced by the MycoTech Corporation. The ability to visualize the distribution of conidia on the insect's exoskeleton was greatly improved by the use of the collodion solution. A minor drawback to this method was the reduced clarity of spores in areas where the collodion strip was thick. The most effective manner to determine the number of viable conidia on each treated weevil was to rinse the weevil with sterile water and plating the rinse on

PDA. Through the results of these studies, the mode and position of ingress was firmly established. It was found that conidia cling to moist areas and condense in intersegmental areas and around the base of hairs and appendages. These data enable us to project the likely source of fungal entry.

Before this study, little was known about whether hemocytes within adult *Diaprepes* root weevil hemolymph served to thwart invasion by *B. bassiana*. The direct observation of fresh, unstained hemolymph under phase interference microscopy established that hemocytes were active in infected insects and were seen actively engulfing hyphal bodies 72 hours post inoculation. It appeared that hemocytes had a limited impact on proliferation of the fungus, because 96 hours post inoculation branching, septate hyphae could be found in female abdominal hemolymph. Within five days following inoculation branched hyphal bodies were found in all body parts examined in both sexes.

It was established that weevil death occurred prior to oviposition, so vertical transmission of *B. bassiana* could not be established. Weevil life span has been measured at 147±17.1 days for females and 135±21.5 days for males (Beavers, 1982). Weevil death from contact with *B. bassiana* inoculum ranged from 6 to 21 days post contact. It is important in the potential use of fungi for biological control to understand mortality as it relates to infection. The majority of infected weevils died between 7 and 10 days. Wolcott (1936) reported that there was a preoviposition period of three to seven days from the time that female weevils emerged from the soil to the time they began to lay eggs. Later it was shown that the preoviposition stage was much longer then previously reported and could last as long as 22 ± 3 days (Beavers, 1982).

An infection system that would inoculate newly emerged weevils prior to reproduction would be particularly effective. Weevils have been shown to touch one another for long periods of time in the wild. In the mock mating in chapter 4 weevils were held in contact for 1 minute, in the wild the conidial transfer rate might be significantly higher because the duration of contact is longer.

What next is needed to establish if *Beauveria bassiana* can be effective as a biological control agent of the *Diaprepes* root weevil? One of the first problems is to get adequate inoculum contact with the weevil under grove and nursery situations. I propose a trapping system based on the modified Tedders trap (Tedders and Wood, 1994; Mizell and Tedders, 1999). A modified citrus Tedders trap is used to monitor adult weevil emergence from the soil and can be used to estimate relative weevil abundance (Duncan et al., 2001). A further modification of this trap would be to create a conidial delivery system for control of *Diaprepes abbreviatus* populations. Although weevil emergence occurs all year there is a peak time for weevil emergence in mid-June with large numbers emerging from the soil until late in September (McCoy et al., 2003). With the information that has been gained in this research, it is foreseeable that use of *Beauveria bassiana* in a modified Tedders trap as a control for the *Diaprepes* root weevil holds promise if timing of applications coincides with adult emergence.

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BIOGRAPHICAL SKETCH

Born and raised in Alachua County, Jennifer Gillett, Florida, youngest child of Michael and Christina Gillett had an interest in science and agriculture from a young age. Always enamored with the outdoors, Jennifer's family could always find her playing with insects and rummaging through plant debris. Jennifer attended high school at Santa Fe High School in Alachua. She was a member of the FFA and was active in many sports. In 1992 she began work as a summer intern for the USDA, the work she did was used as her senior science fair project. After graduation she attended the University of Florida as a freshman in 1993 and began working full time that year for the USDA. As an undergraduate she studied agricultural education and communication with a specialization in entomology.

After graduation in 1998 she joined the Peace Corps where she was stationed as an agricultural volunteer in northern Morocco. Here, she worked on an erosion control project and on a pesticide safety project. When she returned to Florida in 2000 she resumed her work at the USDA with insect biological control and began work on a master's degree in the Department of Plant Pathology. While perusing her master's she worked with Dr. Bill Zettler in his Fundamentals of Plant Pathology course for graduate and undergraduate students. She received her master's degree in 2001 and began her work with Dr. Jim Kimbrough on a Ph.D. In 2002 she received the Jack Fry Award for Excellence in Graduate Student Teaching from the College of Agriculture and a university wide graduate student teaching award. In 2003, she received a teaching award

from the National Association of College Teachers in Agriculture. She continues to teach Fundamentals of Plant Pathology as an instructor and hopes to continue her research with the USDA and her teaching with the University of Florida after her graduation.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James W. Kimbrough, Chair
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 2003

Dean, College of Agricultural and Life Sciences

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